REMARKS

After entry of this amendment, claims 1, 5, 8-11, 13, 15, 18, 19, 29, 32, 47, 49 and 51-54 are pending. New claims 51-54 have been added and find support *inter alia* in the specification at page 65, lines 4-5, page 67, lines 24-26, page 70, lines 18-20, page 72, lines 16-18, page 74, lines 21-23, and page 76, lines 17-19. No new matter has been added.

Claim Rejections – 35 U.S.C. § 103

Claims 1, 5, 8-10, 13, 18, 19, 29, 32, 47 and 49 remain rejected under 35 U.S.C. § 103(a) as being obvious over Lanahan *et al.* (hereinafter "Lanahan") in view of Gan, Grant *et al.* (hereinafter "Grant") and Samuelson *et al.* (hereinafter "Samuelson"). Claims 11 and 15 also remain rejected under 35 U.S.C. § 103(a) as being obvious over Lanahan in view of Gan, Grant, Samuelson, and further in view of Stomp *et al.* (hereinafter "Stomp"). Applicants respectfully traverse the rejections and request for reconsideration and withdrawal of the rejections for the reasons already of record and for the following additional reasons. The prior art references clearly cannot be combined as proposed.

As a preliminary matter, Applicants note that the Office Action does not expressly comment or rebut the points made in the declaration by Dr. Ritte filed under 37 C.F.R. § 132 that was filed with Applicants' response dated November 4, 2009. Express consideration of the declaration by Dr. Ritte on the record is respectfully requested. As explained in MPEP § 2143, all arguments and evidence presented by applicants in favor of patentability must be considered on the record.[1] Dr. Ritte has testified that the claimed subject matter was not predictable; therefore under *KSR* the claimed subject matter would not have been obvious. The current record does not reflect any consideration of the technical points made by Dr. Ritte. For example, his testimony that the stress mechanisms of yeast and plants are different has not been addressed in the Office Action, and instead, the Examiner continues his reliance on Samuelson which does

[1] MPEP 2142 provides in part: "If the examiner determines there is factual support for rejecting the claimed invention under 35 U.S.C. 103, the examiner must then consider any evidence supporting the patentability of the claimed invention, such as any evidence in the specification or any other evidence submitted by the applicant. The ultimate determination of patentability is based on the entire record, by a preponderance of evidence, with due consideration to the persuasiveness of any arguments and any secondary evidence. *In re Oetiker*, 977 F.2d 1443, 24 USPQ2d 1443 (Fed. Cir. 1992)."

not relate to stress genes (it relates to iron reductase genes) and has no demonstrated or explained relevance to improved stress tolerance. Other points made by Dr. Ritte include:

- The prior art does not demonstrate the claimed utilities (¶ 6);
- Grant shows no evidence that GRX expression is upregulated by the stresses recited in the current claims (¶¶ 9-10);
- Stress mechanisms are poorly understood (¶¶ 13-15);
- The stress mechanisms of yeast and plants are different (¶ 16); and
- A person of ordinary skill in the art at the time of the invention would have seen no basis to predict that overexpression of GRX would increase tolerance in a plant to stresses associated with salinity, drought or low temperature (¶ 17).

These points have not been rebutted, are scientifically valid, and are persuasive of the patentability of the current claims.

Moreover, in maintaining the obviousness rejections, the Examiner finds that it would have been obvious for one skilled in the art to overexpress a glutaredoxin in a plant with an expectation to obtain transgenic plants with increased tolerance to salinity, drought, and low temperature stresses. The Examiner's opinion is based on Grant's teaching that the expression of yeast glutaredoxins, GRX1 and GRX2, are increased in response to oxidative stress, heat shock and osmotic shock (the only stresses actually assessed by Grant). Applicants note that the Examiner's reference to "environmental stresses" is rather more broadly than what is supported by Grant's teaching.

The Examiner's rejections under Section 103 are further premised upon predictability of improving tolerance against certain stresses based upon an assumed showing in the prior art of improving tolerance against different stresses (assuming *arguendo* for the moment that yeast results are actually predictive of what will happen in transgenic plants, and that Grant's overexpression findings are actually predictive of conferring protection when overexpressed in plants, both of which Applicants disagree and traverse). This assumed predictability in the art is inconsistent with the earlier findings of unpredictability in the art used to reject Applicants'

claims for non-enablement. The Examiner's attention is respectfully directed to the Office Actions dated January 25, 2007 and November 14, 2007, in which the Examiner rejected the then-pending claims under 35 U.S.C. § 112, first paragraph, for lack of enablement for encompassing improved tolerance to stresses other than salinity, drought, and low temperature stresses. According to the Examiner, undue experimentation would have been required for one skilled in the art to use the claimed methods to increase tolerance of a plant expressing the recited nucleic acid to any type of environmental stress other than those taught in the specification. The Examiner expressly referred to "unpredictability in the art" in the Office Action dated November 14, 2007, at page 6. Applicants acceded to that ground of rejection and amended the claims to recite salinity, drought, and low temperature stresses. For the Examiner to change his view of the predictability/unpredictability of the art in the middle of prosecution of this application, after Applicants have amended the claims, is manifestly unfair and prejudicial. The "state of the art" as of the filing date of this application has not changed during the prosecution of this application.

Accordingly, there would have been no predictability in the prior art of achieving the claimed subject matter with a reasonable expectation of success, as explained in the declaration of Dr. Ritte. Both prior art rejections are thus factually unsupported, contrary to the evidence in the record, and should be withdrawn.

For at least the above additional reasons and for the reasons already of record, it is respectfully submitted that the combined teaching of the cited references does not render the claimed subject matter even *prima facie* obvious. Accordingly, reconsideration and withdrawal of the rejections is respectfully requested.

Separate consideration to new claims 51-54 is respectfully requested. In addition to exhibiting increased tolerance to an environmental stress associated with salinity, drought, and/or low temperature, new claims 51-54 further require that the transgenic plants further exhibit increased biomass production, photosynthetic yield, seed yield, and/or dry matter production as compared to a non-transgenic wild type plant of the same species due to the expression of the ORSRP coding nucleic acid. It is respectfully submitted that, not only the subject matter recited in claims 1, 29, 47 and 49, from which new claims 51-54 depend, respectively, is not obvious in

view of the cited references, the references, alone or in combination, further fail to teach or suggest that expression of an ORSRP coding nucleic acid in a plant would result in an increase in biomass production, photosynthetic yield, seed yield, and/or dry matter production.

Moreover, Applicants submit that such an increase in biomass production, photosynthetic yield, seed yield, and/or dry matter production in the transgenic plants according to the present application is unexpected and surprising, and thus, nonobvious. It is well recognized in the art that transgenic plants showing increased tolerance to an environmental stress do not necessarily exhibit increased yield. In fact, it is described that stress resistant transgenic plants generally exhibit slower growth and reduced biomass and yield due to a decreased growth rate, which is a result of an imbalance in development and physiology of the plant. See e.g., Serrano *et al.*, Scientia Horticulturae, 1999, 78: 261-269 (hereinafter "Serrano"; copy attached). For instance, as demonstrated in Serrano, tobacco plants exhibiting an enhanced salt and drought tolerance due to the overexpression of a TPS1 (trehalose-6-phosphate synthase) gene also exhibited altered phenotype such as loss of apical dominance, stunted growth, lancet-shaped leaves, and some sterility. See e.g., Serrano at page 261, Abstract, page 266, paragraph below Fig. 2, and page 267, 1st full paragraph under section 4 subtitle. According to the authors' observation, the altered phenotype was always coupled with drought tolerance and plants showing more severe morphological alterations had actually the highest drought and salt tolerance. See Serrano at page 266, lines 3-5 under Fig. 2. Thus, in the case of overexpressing a yeast TPS1 gene in plants, for example, increased tolerance to drought and/or salt in plants due to the expression of the transgene does not lead to increased yield.

Similarly, when a cDNA encoding the DREB1A (dehydration response element binding protein 1A) protein was overexpressed in transgenic plants, it is observed that, while the overexpression of the transgene resulted in improved tolerance to various stress conditions, strong overexpression also resulted in severe growth retardation under normal growing conditions. See Kasuga *et al.* (Nature Biotechnology, 1999, 17: 287-291; hereinafter "Kasuga"; copy attached) at page 287, Abstract.

Thus, as evidenced by at least the Serrano and Kasuga references as discussed above, Applicants respectfully submit that transgenic plants showing tolerance to stress conditions do

not necessarily exhibit increased yield, and that increased yield together with stress resistance is clearly unexpected and patentable. For at least this additional reason, separate consideration and allowance of new claims 51-54 is respectfully requested.

CONCLUSION

In view of the above remarks and further in view of the above amendments, Applicant respectfully requests withdrawal of the rejections and allowance of the claims. If any outstanding issues remain, the Examiner is invited to telephone the undersigned at the number given below.

Applicant reserves all rights to pursue the non-elected claims and subject matter in one or more divisional applications, if necessary.

Accompanying this response is a petition for a one-month extension of time with the required fee payment. No further fee is believed due. However, if an additional fee is due, the Director is authorized to charge our Deposit Account No. 03-2775, under Order No. 13311-00012-US from which the undersigned is authorized to draw.

Respectfully submitted,

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Attachments:

1. Serrano *et al.*, Scientia Horticulturae, 1999, 78: 261-269.
2. Kasuga *et al.*, Nature Biotechnology, 1999, 17: 287-291.

ELSEVIER

Scientia Horticulturae 78 (1999) 261–269

SCIENTIA HORTICULTURÆ

Genetic engineering of salt and drought tolerance with yeast regulatory genes

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Abstract

Two yeast regulatory genes involved in stress tolerance, *HAL1* and *TPS1*, have been expressed in transgenic plants. In yeast cells, *HAL1* modulates cation transport and *TPS1* affects sugar metabolism and indirectly stress genes. In addition, *TPS1* encodes trehalose-6-P synthase and is involved in trehalose synthesis. Transgenic plants expressing *HAL1* exhibited an improved salt tolerance under 'in vitro' culture conditions. In the case of *TPS1*, transgenic plants exhibited several morphological alterations and enhanced salt and drought tolerance. No significant trehalose accumulation was observed in *TPS1*-expressing plants. The results can be explained by activation of endogenous plant defence genes and, in the case of *TPS1*, of other plant responses triggered by the yeast regulatory genes.

Keywords: *HAL1*; Trehalose; Transgenic plants; Osmotic stress; Salt stress; Drought tolerance; Genetic engineering; Yeast; Regulatory genes

Contents

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0304-4238/99/$ – see front matter
PII: S0304-4238(98)00196-4

1. Regulation of salt and drought-stress responses in yeast and plants

Salinity and drought pose to plants a similar problem of water deficit and in both stresses the hormone abscisic acid plays an important regulatory role (Bray, 1997). The response of plants to salt and drought stress is based on the transcriptional activation of a plethora of putative defence proteins whose participation in stress tolerance is only partially understood. In addition to osmotic stress, high salinity also leads to ion toxicity effects, but specific responses to this form of stress are highly quite unknown (Serrano, 1996). Inducibility by osmotic stress, and in many cases by abscisic acid, is the only correlative evidence for a putative role in stress tolerance of most stress proteins (Chandler and Robertson, 1994; Serrano and Gaxiola, 1994; Bray et al., 1996). Lack of biochemical studies with the purified proteins and the absence of mutants with gain or loss of function precludes the assignment of defined functions to most of the putative defence proteins. A further complication is the lack of specificity of plant responses to stress, as demonstrated by the salt induction of heat shock proteins, antioxidative damage enzymes, antifungal proteins and inhibitors of insect digestion (Serrano and Gaxiola, 1994; Ingram and Bartels, 1996). Therefore, the utilisation of genes encoding salt- and drought-induced proteins of unknown function to engineer salt tolerance in transgenic plants has many uncertainties and, with a single exception (Xu et al., 1996), has produced no useful results.

Osmolyte synthesis is one of the few stress responses which has been studied by biochemical and genetic approaches and transgenic plants with increased osmotic tolerance have been engineered with genes encoding osmolyte synthetic enzymes. This approach is based on the assumption that osmolyte synthesis is a limiting factor in drought and salinity tolerance and is discussed by Hans J. Bohnert and Bo Shen in this issue. We will discuss here an alternative approach based on the expression in transgenic plants of regulatory proteins involved in yeast responses to salinity.

The utilisation of the yeast *Saccharomyces cerevisiae* as a model system in the field of osmotic and salt stress has provided a novel perspective. A genetic analysis has started to define the crucial reactions involved in salt stress tolerance and several signal transduction components, such as protein kinases and phosphatases, have been identified as limiting factors in salt tolerance (Serrano, 1996; Serrano et al., 1997). Starting from these premises, we formulated the

hypothesis that the regulatory components of stress signalling pathways may also be limiting in plant stress tolerance. A further assumption was that stress signal transduction pathways may be conserved between yeast and plants. Accordingly, yeast stress regulators may enhance plant defence responses by triggering the expression of unidentified endogenous plant defence proteins. In this way, a pleiotropic defence response involving several endogenous plant genes could be induced with a single transgene. This approach could make unnecessary the transfer of multiple genes for tolerance engineering as recently advocated (Bohnert and Jensen, 1996). The first successful results of this approach, involving the yeast *HAL1* and *TPS1* regulatory genes, are described below.

2. Transgenic melon expressing the yeast *HAL1* gene

2.1. Mechanism of action of HAL1 in yeast

The yeast *HAL1* gene was originally isolated in a screening for genes which upon overexpression in multicopy plasmids increased salt tolerance in the yeast *Saccharomyces cerevisiae* (Gaxiola et al., 1992). *HAL1* encodes a soluble, cytoplasmic protein, still without significant homologies in data banks. *HAL1* is a potent regulator of ion homeostasis, modest overexpression from its own promoter mostly affected intracellular potassium levels (Gaxiola et al., 1992) while more drastic overexpression from a strong promoter affected both potassium and sodium homeostasis (Rios et al., 1997). Overexpression of *HAL1* reduces K^+ loss from salt-stressed cells, a phenomenon mediated by an unidentified K^+ efflux system. The effect on sodium levels is based on increased expression of the sodium-extrusion pump encoded by the *ENA1* gene. Accordingly, *HAL1* overexpressing cells maintain a higher intracellular K^+ level, a lower intracellular Na^+ level and a higher K^+/Na^+ ratio than control cells, the latter probably explaining the improvement in salt tolerance (Serrano, 1996). How a soluble cytoplasmic protein like the product of the *HAL1* gene modulates transport systems for K^+ and Na^+ efflux is at present unknown. The protein probably participates in an unidentified signal transduction pathway for ion homeostasis. Despite lack of knowledge about its mechanism of action, *HAL1* has a strong halotolerance phenotype in yeast and therefore it was chosen for our first trial of yeast regulatory genes expressed in transgenic plants.

2.2. Evaluation of halotolerance in transgenic melon plants expressing the yeast HAL1 gene

The availability of an efficient plant regeneration system from explant-derived calli of melon (Garcia-Sogo et al., 1991) allowed us to generate large numbers of

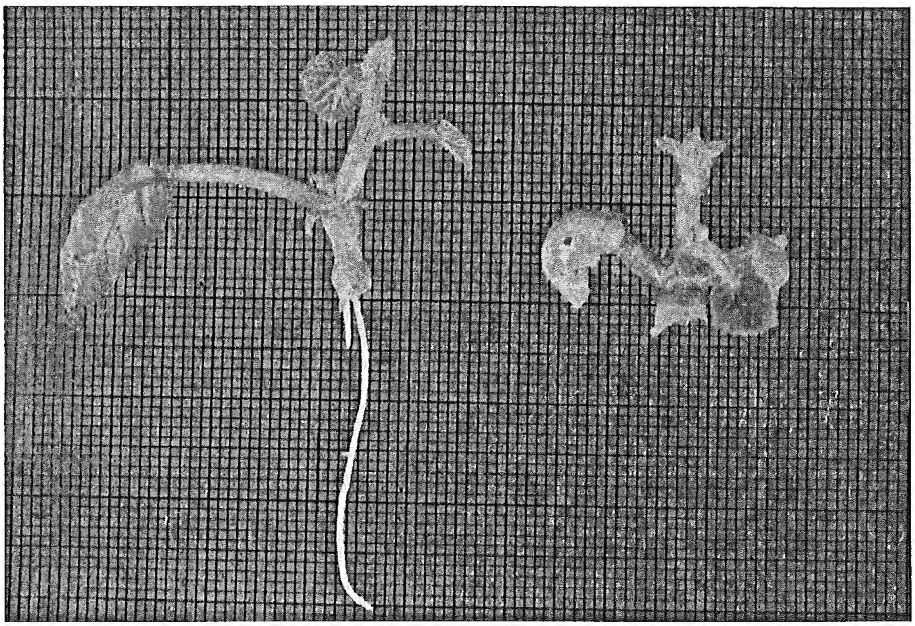

Fig. 1. Rooting, in high salt medium, of apical shoots from control (right) and transgenic (left) melon plants expressing the yeast *HAL1* gene.

transgenic melon plants expressing the yeast *HAL1* gene (Bordás et al., 1997; see Fig. 1). Cotyledon and leaf explants of cv. Pharo were used in these experiments of Agrobacterium-mediated transformation and, eventually, two sets of transgenic plants were obtained: 65 from cotyledons and 75 from leaf explants. Two kinds of controls were included: cotyledon-derived untransformed plants and escapes (*HAL1* negative plants from transformation experiments).

The evaluation of the halotolerance level in primary transformants was performed by using a shoot growth test (Bordás et al., 1997). Apical shoots (2 cm long) were excised from 2-week-old plants and transferred to medium containing 10 g l^{-1} NaCl. Control plants produced no roots under these conditions (less than 2% of control apical shoots rooted). On the other hand, the two populations of transgenic plants rooted on this medium with a frequency of 15–20%. The better performance of transgenic plants exposed to a relatively high level of NaCl is most probably due to the effect of the *HAL1* gene. Epigenetic or genetic variations induced by the transformation procedure can, in principle, be discarded because controls have experienced exactly the same treatments as the transgenic plants.

It is important to point out that the results of evaluation of some progenies obtained by selfing primary *HAL1*-transformants of melon (C. Roig and V. Moreno, in preparation) indicate that salt tolerance conferred by *HAL1* is sexually

transmitted. In parallel experiments we have also transferred the *HAL1* gene to both tomato (C. Gisbert, C. Montesinos, R. Serrano and V. Moreno, in preparation) and watermelon (P. Ellul and V. Moreno, in preparation) and the results of the evaluation of salt tolerance by 'in vitro' culture indicate that *HAL1* conferred salt tolerance also in these species. At present, a progeny derived from a tomato transgenic plant and two derived from watermelon transgenic plants are being evaluated using an hydroponic culture system. These experiments will give an evaluation of *HAL1* under conditions closer to agricultural practice than in vitro tests.

3. Transgenic tobacco expressing the yeast *TPS1* gene

3.1. Metabolic and regulatory roles of the TPS1 gene in yeast

The *TPS1* gene of *Saccharomyces cerevisiae* encodes trehalose-6-phosphate synthase, a protein which can be considered both as a metabolic enzyme and as a regulator. In addition to its role in trehalose biosynthesis, the product of the *TPS1* gene is involved in controlling sugar influx into glycolysis and sugar-induced signalling (Bell et al., 1992; Gonzalez et al., 1992; de Winde et al., 1997). In *tps1* mutants sugar-phosphates accumulate to very high levels in the presence of glucose, glycolysis stops because of depletion of ATP and Pi and several glucose-induced signalling phenomena such as increase of cAMP, inactivation of fructose-1,6-bisphosphatase and activation of glycolytic enzymes are impaired (de Winde et al., 1997). The most likely mechanism is the modulation of the sugar-phosphate pool by a futile cycle of trehalose synthesis and hydrolysis. *TPS1* seems to modulate the heat shock response positively in yeast (Hazell et al., 1995) and therefore it could be a general stress regulator. The expression in transgenic plants of the yeast *TPS1* gene was attempted with two different purposes. In the first place, trehalose accumulation could be triggered and the osmolyte role of this sugar could result in stress protection. On the other hand, it is possible that the high activity of trehalase in plant tissues (Müller et al., 1995) prevents trehalose accumulation but that the futile cycle of trehalose synthesis and degradation depletes the sugar-phosphate pool and up-regulates defence responses. As discussed below, this second possibility provides a credible explanation of the experimental results obtained with transgenic tobacco.

3.2. Pleiotropic phenotypes of transgenic tobacco-expressing yeast TPS1: Osmolyte accumulation and perturbation of carbohydrate metabolism

As shown in Fig. 2, the expression of the yeast *TPS1* gene in tobacco produced significant improvements in drought and salt tolerance (Romero et al., 1997). A

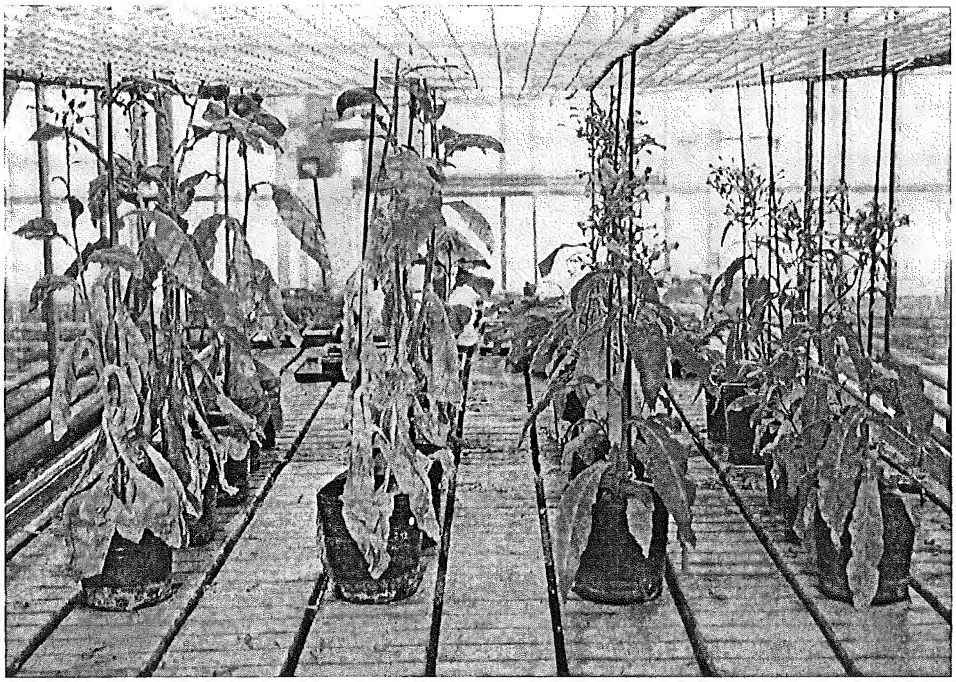

Fig. 2. Drought tolerance of transgenic tobacco plants expressing the yeast *TPS1* gene. The two left roaws contain non-transgenic controls and the two right rows contain F_2 homozygotic transgenic plants. All the plants have been deprived of water for 15 days. Essentially similar results are obtained by irrigation for 15 days with 400 mM NaCl. The better tolerance and smaller size of the transgenic plants is apparent.

large fraction (40%) of F_0 trehalose-accumulating plants exhibited different degrees of phenotypic changes, related to loss of apical dominance, stunted growth, lancet-shaped leaves, and some sterility. Altered phenotype was always coupled with drought tolerance and those plants showing more severe morphological alterations had the highest drought and salt tolerance. Phenotypic alterations and drought tolerance segregate as linked characters in F_1 plants. These results are in agreement with those reported by Goddijn et al. (1997) but do not support the assertion of Holmström et al. (1996) that "Trehalose accumulation caused no obvious morphological changes in self-pollinated progeny of *TPS1* positive plants."

The measured concentrations of trehalose in the transgenic plants (<0.5 mM) are too low for a conventional osmoprotectant effect. Thus, the relatively low trehalose concentrations in desiccation-tolerant angiosperms (Müller et al., 1995) and in transgenic plants expressing trehalose-phosphate synthase, suggest that trehalose may play a more complex role in preventing plant desiccation than just contributing to osmotic adjustment, a function that could be performed by other

more abundant functional analogues like sucrose. Trehalose synthesis may affect metabolic and hormonal pathways leading to pleiotropic changes including stress tolerance. The carbohydrate profile differed between control and transgenic plants (Romero et al., 1997), suggesting changes in basic biochemical pathways such as carbohydrate metabolism. Work is in progress to check if the morphological and biochemical alterations produced by the *TPS1* gene are linked with plant stress response pathways.

4. Stress tolerance with heterologous regulators: Common themes in signal transduction or useful artefacts?

Two aspects of the work described above deserve discussion. One is the fact that the phenotype of increased stress tolerance in transgenic plants is sometimes accompanied by decreased growth rate. This is apparent in the case of transgenic tobacco expressing the yeast *TPS1* gene. Recently, the salt tolerance of transgenic tobacco accumulating mannitol has been ascribed to slower growth resulting in reduced salt accumulation (Karakas et al., 1997). It is not clear if this mechanism could explain drought tolerance. In the case of transgenic melon expressing the yeast *HAL1*, however, growth retardation is not observed and therefore this simple explanation cannot apply.

The other point refers to the mechanisms of action of heterologous regulatory proteins. Potentiation of endogenous plant defences by the transgenes is a plausible mechanism to explain the stress tolerance conferred by *HAL1* and *TPS1*. Although *TPS1* in yeast may have general effect on stress tolerance (Hazell et al., 1995), *HAL1* in yeast is clearly restricted to sodium stress (Rios et al., 1997). It would be interesting to determine if the transgenic plants expressing *HAL1* are also tolerant to other stresses in addition to salinity. Also, it would be important to determine if the drought-tolerant plants expressing *TPS1* are also tolerant to other stresses. Finally, the potentiation of endogenous defences should be examined by analysing the expression levels of different plant defence genes.

Clearly, we cannot yet answer the question of whether the stress tolerance achieved with heterologous regulators reflects either common themes between yeast and plants or useful artefacts. Our knowledge of stress signal transduction pathways both in yeast and plants is still too fragmentary to determine if *HAL1* and *TPS1* perform similar functions in both types of organisms. In any event, the positive results described in the present work may be of biotechnological relevance and suggest that in addition to genes corresponding to direct defence against stress, regulatory genes may also be useful in improving stress tolerance.

Other yeast regulatory genes which could be tested in transgenic plants include the protein phosphatases calcineurin (Nakamura et al., 1993) and PPZ (Posas et al., 1995) and the protein kinases of the HOG-MAP kinase pathway (Brewster

et al., 1993; Maeda et al., 1994). Overexpression of calcineurin has been shown to confer salt tolerance in yeast (Mendoza et al., 1996) but increased activity of HOG-MAP kinase is toxic for yeast cells (Maeda et al., 1994). Therefore, it is difficult to predict the consequences of the expression of these yeast regulators in transgenic plants.

In addition to yeast, other sources of novel regulatory and defence genes with potential uses in plant stress tolerance could be halophytic microorganisms such as *Candida tropicalis* (Garcia et al., 1997) and *Dunaliella* (Pick, 1992) and halophytic (Flowers et al., 1977) and dehydration tolerant (Ingram and Bartels, 1996) plants.

Acknowledgements

Work in the author's laboratories has been supported by grants of the European Union (Brussels, AIR3-CT94-1508 and BIO2-CT93-0400), of the spanish 'CICYT" (Madrid, BIO96-1196-CO2) and of the "Conselleria de Agricultura de la Generalitat Valenciana" (Valencia).

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Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor

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Received 2 July 1998; accepted 4 January 1999

Plant productivity is greatly affected by environmental stresses such as drought, salt loading, and freezing. We reported previously that a *cis*-acting promoter element, the dehydration response element (DRE), plays an important role in regulating gene expression in response to these stresses. The transcription factor DREB1A specifically interacts with the DRE and induces expression of stress tolerance genes. We show here that overexpression of the cDNA encoding DREB1A in transgenic plants activated the expression of many of these stress tolerance genes under normal growing conditions and resulted in improved tolerance to drought, salt loading, and freezing. However, use of the strong constitutive 35S cauliflower mosaic virus (CaMV) promoter to drive expression of *DREB1A* also resulted in severe growth retardation under normal growing conditions. In contrast, expression of *DREB1A* from the stress inducible *rd29A* promoter gave rise to minimal effects on plant growth while providing an even greater tolerance to stress conditions than did expression of the gene from the CaMV promoter.

Keywords: drought tolerance, freezing tolerance, transgenic plants, multigene expression, stress-inducible promoter

Drought, salt loading, and freezing are stresses that cause adverse effects on the growth of plants and the productivity of crops. The physiologic response to these stresses arises out of changes in cellular gene expression. Expression of a number of genes has been demonstrated to be induced by these stresses[1,2]. The products of these genes can be classified into two groups: those that directly protect against environmental stresses and those that regulate gene expression and signal transduction in the stress response[3]. The first group includes proteins that likely function by protecting cells from dehydration, such as the enzymes required for biosynthesis of various osmoprotectants, late-embryogenesis-abundant (LEA) proteins, antifreeze proteins, chaperones, and detoxification enzymes[3–5]. The second group of gene products includes transcription factors, protein kinases, and enzymes involved in phosphoinositide metabolism[3].

Recently, several different gene transfer approaches have been employed to improve the stress tolerance of plants[6]. The transferred genes included those encoding enzymes required for the biosynthesis of various osmoprotectants[7–9] or those encoding enzymes for modifying membrane lipids[10,11], LEA protein[12], and detoxification enzyme[13]. Each of these experiments involved transfer of a gene encoding a single specific stress-protective protein, expressed from the 35S cauliflower mosaic virus (CaMV) promoter. To investigate the possibility of simultaneously enhancing tolerance toward multiple stresses (i.e., drought, salt loading, and freezing) by gene transfer, we transferred a gene encoding a stress-inducible transcription factor that regulates many genes involved in stress tolerance in *Arabidopsis thaliana*.

Analyses of the expression of dehydration-inducible genes in *Arabidopsis* have indicated that at least four independent signal pathways function in the induction of stress-inducible genes in response to dehydrastion[3]. Two are abscisic acid (ABA)-dependent and two are ABA-independent. One of the two ABA-independent pathways overlaps with that of the cold-response. Several stress-induced genes, such as *rd29A*, are induced through this separate ABA-independent pathway[14–18]. A *cis*-acting element has been identified in the promoter region of the *rd29A* gene and is responsible for dehydration- and cold-induced expression[19]. This sequence (TACCGACAT), termed the dehydration-responsive element (DRE), is essential for the regulation of dehydration-responsive gene expression[19] and is found in the promoter regions of other dehydration- and cold-stress inducible genes[18,20]. The cDNAs encoding the DRE-binding proteins, DREB1A and DREB2A, have been isolated by yeast one-hybrid screening[21]. Both proteins specifically bind and activate transcription of genes containing the DRE sequence in *Arabidopsis*.

Expression of the *DREB1A* cDNA under control of the 35S CaMV promoter in transgenic plants gave rise to strong constitutive expression of the stress-inducible genes, and increased tolerance to freezing, salt, and drought stresses[21]. A cDNA encoding CRT/DRE binding protein (CBP1) was isolated from *Arabidopsis* and when overexpressed also enhanced freezing tolerance[22,23]. However, the overexpression of these genes resulted in severe growth retardation under normal growth conditions. Here, we use the stress-inducible *rd29A* promoter to drive expression of *DREB1A*, with the aim of minimizing the negative effects on plant growth experienced with use of the 35S CaMV promoter. We observed improved stress tolerance of the transgenic plants and much improved growth under nonstressed conditions.

Results

Preparation of transgenic *Arabidopsis*. *Arabidopsis* (Columbia ecotype) plants were transformed with vectors expressing the DREB1A cDNA from either a modified 35S CaMV promoter[24] or the stress-inducible *rd29A* promoter. Eighteen antibiotic-resistant *Arabidopsis*

transformants carrying the 35S:DREB1A transgene (35S:DREB1A plants) and 43 transformants carrying the *rd29A:DREB1A* transgene (*rd29A*:DREB1A plants) were generated using a vacuum infiltration method[25].

Analyses of the 35S:DREB1A plants. Growth of the 35S:DREB1A transformants was compared with wild-type control plants after 35 (Fig. 1A) and 53 days (Fig. 1B). The transgenic plants demonstrated varying degrees of growth retardation, which we postulated to be due to variation in expression of the *DREB1A* transgene[21]. Three of the 18 transformants showed severe growth inhibition, and were classified as 35S:DREB1Aa plants. Transformants exhibiting lesser degrees of growth retardation were classified as either 35S:DREB1Ab (moderate phenotypic changes) or 35S:DREB1Ac (mild phenotypic changes) plants. The growth retardation affected seed numbers under normal growth (control) conditions. We observed a significant reduction in the number of seeds produced by the 35S:DREB1Aa and 35S:DREB1Ab plants compared with wild-type plants. The number of seeds harvested from the 35S:DREB1Aa plants was less than 1% of the wild-type controls. In contrast, the 35S:DREB1Ac plants produced a number of seeds similar to wild-type plants.

The expression of *DREB1A* and its target genes was analyzed in 35S:DREB1Ab and 35S:DREB1Ac plants and compared with control plants transformed with the pBI121 vector (Fig. 2). In the 35S:DREB1Ab plants, the *kin1, cor6.6/kin2, cor15a, cor47/rd17,* and *erd10* stress-response genes[3,26] were strongly expressed under control conditions, as was the *rd29A* gene. The expression of these genes in the 35S:DREB1Ac plants was only slightly higher than in wild-type plants but was noticeably lower than in 35S:DREB1Ab plants under unstressed conditions. In contrast, we detected no difference in the expression of the stress-inducible *AtP5CS, erd1, rd22,* and *rd29B* genes[14,27–29] between the 35S:DREB1A and wild-type plants. These results indicate that overexpression of the DREB1A protein leads to specific induction of its target stress-response genes, but not of nontarget stress-response control genes under conditions in the 35S:DREB1A plants, and that the level of stress-responsive gene expression correlates with levels of *DREB1A* mRNA.

Analyses of the *rd29A*:DREB1A plants. *rd29A*:DREB1A plants were then examined under growth conditions. The *DREB1A* cDNA was driven by the strong stress-inducible *rd29A* promoter. Nearly all of the *rd29A*:DREB1A plants exhibited slight growth retardation under control conditions (Fig. 3). However, unlike the 35S:DREB1A plants, there were no significant differences in growth retardation between the transgenic *rd29A*:DREB1A plant lines. Moreover, seed numbers for the *rd29A*:DREB1A transformants were similar to wild-type plants grown under control conditions.

The expression pattern of the *DREB1A* and *rd29A* genes was similar between the various *rd29A*:DREB1A transformant plants. Expression of these genes in transgenic plant *rd29A*:DREB1Aa is shown in Figure 4. The *DREB1A* transgene was expressed at levels above the endogenous gene in wild-type plants even under control conditions, yet was strongly induced by dehydration, salt, cold stress, and treatment with ABA in the *rd29A*:DREB1Aa plants. Under control conditions, expression of the *DREB1A* gene in the *rd29A*:DREB1Aa plants was much less than in the 35S:DREB1Aa plants and similar to that in the 35S:DREB1Ac plants (Fig. 4). In contrast, we detected similar strong expression of the *DREB1A* gene in both the *rd29A*:DREB1Aa and 35S:DREB1Aa plants under conditions of stress (dehydration, salt, low temperature, and treatment with ABA). We observed similar patterns of expression of the *rd29A* gene in the *rd29A*:DREB1Aa plants. The *rd29A* gene was expressed at low levels under control conditions and was strongly induced by each of the stress treatments. A similar pattern of expression was observed for other stress-inducible, DREB1A-responsive genes in the *rd29A*:DREB1A transgenic plants (data not shown).

Freezing, drought, and salt stress tolerance of the transgenic plants. The tolerance of the *rd29A*:DREB1A plants to freezing, dehy-

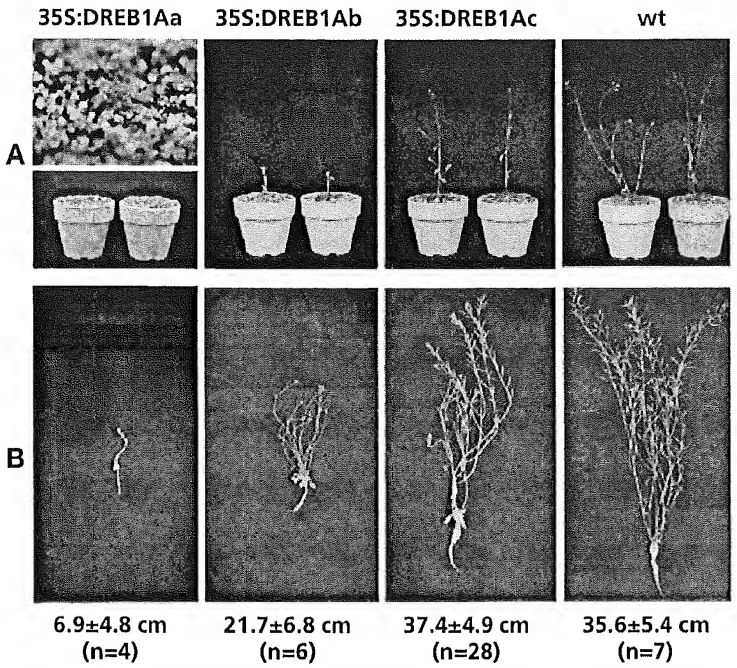


Figure 1. Phenotypes of the 35S:DREB1Aa, 35S:DREB1Ab, and 35S:DREB1Ac plants in relation to wild-type plants (pBI121). (A) Plants grown for 35 days. (B) Plants grown for 53 days. The average height of each plant line grown for 53 days is indicated under each picture.

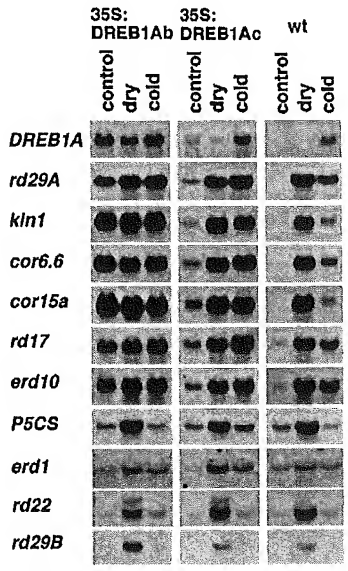


Figure 2. Expression of *DREB1A* target gene mRNAs in 35S:DREB1A transgenic plants and in the wild-type controls. Each lane was loaded with 20 µg of total RNA prepared from transgenic *Arbidopsis* plants that had been dehydrated for 5 h (dry), treated at 4°C for 5 h (cold), or left untreated (control). The DNA fragments of the full-length *DREB1A, KIN1, COR6.6, cor15a, RD17, ERD10, AtP5CS, ERD1,* and *RD22* cDNAs and the 3′-terminal specific DNA fragment of *rd29A* and *rd29B* were used as probes.

dration, and salt stress was compared with that of the 35S:DREB1Ab and 35S:DREB1Ac plants grown in pots or on agar plates at 22°C for 3 weeks (Fig. 5A). When plants grown in pots were exposed to temperatures of -6°C for 2 days, then returned to 22°C and grown for 5 days, less than 10% of the wild-type plants survived, whereas 77.9% and 96.2% of the 35S:DREB1Ab and *rd29A*:DREB1Aa plants survived, respectively (Table 1A). The surviving transgenic plants continued to grow and subsequently flowered. Freezing tolerance of the 35S:DREB1A plants correlated with the level of expression of the stress-inducible DREB1A-responsive genes under control conditions: fewer of the 35S:DREB1Ac plants survived than 35S:DREB1Ab plants (48.8% vs. 77.9% survival, respectively; Table 1A). In contrast, the freezing tolerance of the *rd29A*:DREB1Aa plants was much stronger than that of the 35S:DREB1Ab plants, even though the expression of the stress-inducible DREB1A-responsive genes was weak under control conditions (Fig. 5A and B).

To test whether overexpression of the *DREB1A* gene enhanced tolerance to dehydration, the wild-type and transgenic plants grown in pots were not watered for 2 weeks (Fig. 5A). Nearly all the wild-type plants died within this 2-week period, whereas 69.2% of the 35S:DREB1Ab transgenic plants survived this level of drought stress and continued to grow when watering resumed (Table 1B). The toler-

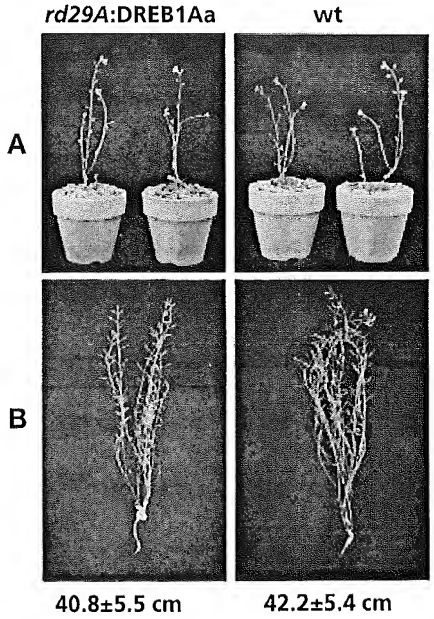


Figure 3. Phenotypes of the *rd29A*:DREB1Aa and wild-type control plants (transformed with the vector pBI121) under control conditions. (A) Plants grown for 30 days. (B) Plants grown for 63 days. The average height of each plant line grown for 63 days is indicated under each picture.

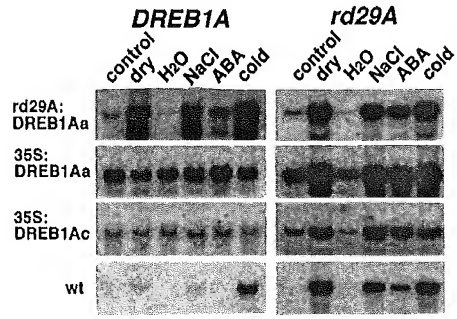
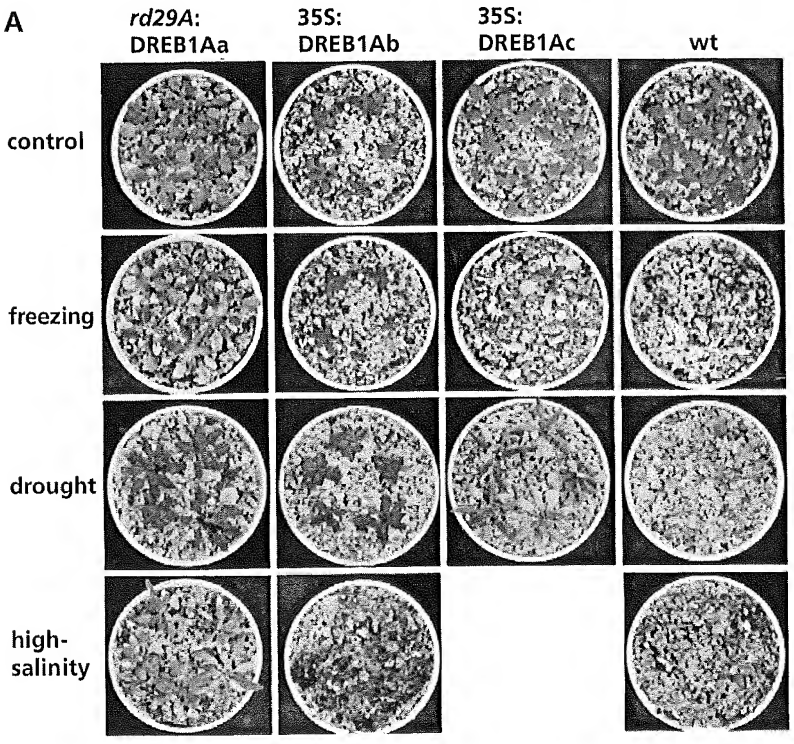


Figure 4. Expression of the *DREB1A* and *rd29A* genes in 35S:DREB1A and *rd29A*:DREB1A transgenic plants. Each lane was loaded with 20 µg of total RNA prepared from transgenic *Arbidopsis* plants that had been dehydrated (dry), grown hydroponically in 250 mM NaCl (NaCl), in water (H_2O), or in 100 µM ABA (ABA), or transferred to agar plates at 4°C (cold), or untreated (control). The stress treatments were continued for 5 h.



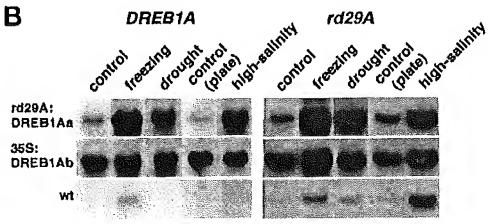


Figure 5. (A) Freezing, drought, and salt stress tolerance of the 35S:DREB1Ab, 35S:DREB1Ac, and *rd29A*:DREB1Aa transgenic plants. The stress treatments were performed as described in the text. Control: 3-week-old plants growing under normal conditions; freezing: plants exposed to a temperature of -6°C for 2 days and returned to 22°C for 5 days; drought: water withheld for 2 weeks; high-salinity: plants soaked in 600 mM NaCl solution for 2 h and transferred to pots under control conditions for 3 weeks. (B) Expression of the *DREB1A* and *rd29A* genes in the 35S:DREB1A and *rd29A*:DREB1A transgenic plants subjected to freezing, drought, and salt stress. Control: 3-week-old plants growing under normal conditions; freezing: plants exposed to a temperature of -6°C for 2 days; drought: water withheld for 5 days; control: plants grown on GM agar plates for 2 weeks; high-salinity: plants that were grown on agar plates for 2 weeks, soaked in 600 mM NaCl solution for 2 h.

Table 1. Survival rates of transgenic plants.

A. Freezing tolerance

	Survival	Total	%
rd29A:DREB1A	177	184	96.2
35S:DREB1Ab	67	86	77.9*
35S:DREB1Ac	40	82	48.8*
wt	9	95	9.5*

B. Drought tolerance

	Survival	Total	%
rd29A:DREB1A	92	120	76.7
35S:DREB1Ab	45	65	69.2
35S:DREB1Ac	23	58	39.7*
wt	1	55	1.8*

C. High-salinity stress tolerance

	Survival	Total	%
rd29A:DREB1A	125	159	78.6
35S:DREB1Ab	10	34	29.4*
wt	7	39	17.9*

Number of plants surviving exposure to (A) freezing, (B) drought, and (C) high-salinity. Experiments were repeated five times. Five to fifty plants were tested in each experiment. Statistical significance, compared with the value of *rd29A*:DREB1A, was determined by χ^2 test (*$p<0.005$).

ance of the 35S:DREB1A plants to drought stress depended on the level of the expression of DREB1A-responsive genes under control conditions: 39.7%, 69.2%, and 76.7% survival was observed for the 35S:DREB1Ac, 35S:DREB1Ab, and *rd29A*:DREB1A plants, respectively (Table 1B). To examine the tolerance of the transgenic plants to salt stress, plants grown on agar plates were removed from the plates, soaked in 600 mM NaCl solution for 2 h, and then grown in pots for 3 weeks (Fig. 5A). Only 17.9% of the wild-type plants survived this treatment compared with 29.4% of the 35S:DREB1Ab plants (Table 1C). In contrast, the *rd29A*:DREB1A plants were highly tolerant of the salt stress (78.6% survival; Table 1C).

The expression of *DREB1A* and DREB1A-responsive genes was examined in the transgenic plants after freezing, drought, and high-salinity stress treatments (Fig. 5B). The *DREB1A* and *rd29A* genes were weakly expressed even under control conditions in the *rd29A*:DREB1Aa plants. Upon exposure to stress conditions, expression of these genes was elevated to similar levels in *rd29A*:DREB1Aa to that in 35S:DREB1Ab plants (Fig. 5B). These results indicate that strong expression of the DREB1A-responsive genes under stress conditions correlated with tolerance to freezing, drought, and high-salinity stress.

Discussion

Many genes have been demonstrated to respond to drought, high salt levels, and cold stress, and the proteins encoded by these genes are thought to function in protecting cells from these stresses. In the present study, we were able to produce transgenic plants that were highly drought-, salt-, and freezing-tolerant by overexpressing a single gene for a stress-inducible transcription factor, DREB1A. DREB1A binds to the *cis*-acting DRE and regulates the expression of many stress-inducible genes under drought, salt, and cold stress in *Arabidopsis*[21]. The expression of the transferred *DREB1A* gene and its stress-inducible target genes were correlated in the transgenic plants under control conditions. We detected the overexpression of six stress-inducible DREB1A-responsive genes in both 35S:DREB1A and *rd29A*:DREB1A transgenic plants. The genes encoding proteins involved in stress tolerance seem to be overproduced even under control conditions in the 35S:DREB1A transgenic plants, but are rapidly overproduced only in response to stress treatments in the *rd29A*:DREB1A plants. These target genes (*rd29A, kin1, cor6.6/kin2, cor47/rd17, cor15a,* and *erd10*) contain the DRE or related motifs[18–20,22], and are normally induced by dehydration, salt, and cold stresses. In contrast, the *AtP5CS, erd1, rd22,* and *rd29B* genes, which do not contain the DRE or related sequences[27–30] and are not targets of DREB1A, were not overexpressed in *DREB1A* transgenic plants. However, we believe that additional stress-inducible genes may be overexpressed in these transgenic plants under control conditions.

LEA proteins appear during the maturation of embryos and desiccation of seeds, and are also induced by drought, salt, and cold stresses in the vegetative tissues of various plants[31,32]. These proteins are quite hydrophilic and are believed to function by directly protecting plant cells from these stresses. One stress-inducible DREB1A-responsive gene, *rd29A,* encodes a protein similar to the LEA proteins[14]. The *cor47/rd17* and *erd10* genes both encode group 2 LEA proteins[31]. The *kin1* and *cor6.6/kin2* genes encode proteins that are structurally similar to the alanine-rich antifreeze proteins produced by some fish[20]. These similarities in structure and expression suggest that the products of these genes may have similar functions in plants. The *cor15a* gene encodes a protein that is targeted to the stromal compartment of the chloroplasts[33], and enhances the freezing tolerance of *Arabidopsis* leaf protoplasts[34].

Cold acclimation increases the freezing tolerance of plants[2]. Various genes are induced during cold acclimation, such as *rd29A/cor78/lti78, kin1, cor6.6/kin2, cor15a, cor47/rd17,* and *erd10.* The DRE is involved in the induction of these genes in response to low temperatures[1]. Overexpression of *DREB1A* induced expression of these genes under control conditions and increased the freezing tolerance of transgenic plants in a fashion reminiscent of what occurs during cold-acclimation of wild-type plants. Overexpression of CBF1, a DREB1A homolog, both enhanced freezing-stress tolerance and increased the expression of *cor15a, cor6.6,* and *cor47* (ref. 23*)*. These observations demonstrate that the target genes of both the DREB1A and CBF1 encode proteins that function in protecting cells from freezing stress. Overexpression of DREB1A also enhanced drought and salt tolerance in the transgenic plants, demonstrating that the proteins encoded by the target stress-inducible genes also function in protecting cells from drought and salt stress.

The 35S:DREB1A plants exhibited varying degrees of growth retardation under control conditions. The extent of both growth retardation under control conditions and stress tolerance correlated with the level of constitutive expression of the DREB1A transcript and its target genes. These results indicate that stress tolerance of the 35S:DREB1A plants comes at the expense of growth and productivity.

To overcome the problem of growth retardation, we used the stress-inducible *rd29A* promoter to overexpress DREB1A. The *rd29A* promoter is stress-inducible and contains binding sites for the DREB1A protein. This promoter allowed low levels of DREB1A expression during unstressed conditions, yet permitted rapid high-level expression of the DREB1A transgene during exposure to dehydration, salt, and low-temperature stress. Thus, strong stress tolerance was accomplished without significant growth retardation under control conditions. Indeed, the growth and seed production of these plants was similar to wild-type plants under normal growing conditions. *rd29A*:DREB1A plants are more tolerant to stress than the 35S:DREB1A plants. As the *rd29A* gene is one of the target genes of the DREB1A protein, the *rd29A* promoter is highly suited to the tissue-specific expression of DREB1A. In *rd29A*:DREB1A plants, the target gene products appear to accumulate in the same tissues that express the stress-inducible genes during stress conditions. These results indicate that transgenic expression of the DREB1A cDNA from the *rd29A* promoter should be quite useful for improving drought, salt, and

freezing-stress tolerance in plants. Previously, we showed that the DRE also functions in stress response in tobacco plants[14,19], which suggests the existence of similar regulatory systems in tobacco and other crop plants. DRE-related motifs have been reported in the promoter region of cold-inducible *Brassica napus* and wheat genes[35,36]. These observations suggest that both the DREB1A cDNA and the *rd29A* promoter can be used to improve the dehydration, salt, and freezing tolerance of agriculturally important crops by gene transfer.

Experimental protocol

Overexpression of *DREB1A* in transgenic plants. The 35S:DREB1A plasmid was constructed as described previously[20]. To construct *rd29A*:DREB1A, a *Bam*HI fragment of the *DREB1A* cDNA was cloned into the *Bam*HI site of the pBI29APNot. pBI29APNot was constructed by ligation of the *Hind*III fragment of the *rd29A* promoter (-861 to +63) into the *Hind*III site of pBI101 (Clontech, Palo Alto, CA). The *Hind*III fragment of the *rd29A* promoter was amplified by PCR with the primers, 5′-AAGCTTGCCATAGATGCAATTAATC-3′ and 5′-AGCTTTTGGAAAGATTTTTTTCTTTCCAA-3′. The resulting plasmid was digested with *Sma*I and *Sac*I to delete the β-glucuronidase coding region, and ligated to a *Sma*I-*Not*I-*Sac*I polylinker (Takara, Tokyo, Japan). The constructs were introduced into *Agrobacterium tumefaciens* C58 as described previously[19]. The *Arabidopsis* plants selected for transformation were grown under continuous illumination in 9 cm pots at approximately 2500 lux and 22°C for 6 weeks. Plants were transformed by the vacuum infiltration method[22].

Phenotypes of the transgenic plants. The transgenic 35S:DREB1A and *rd29A*:DREB1A plants, which were grown on germination medium (GM) agar plates containing kanamycin (30 mg/L) for 7 days, were transferred to 9 cm pots filled with a 1:1 mixture of perlite and vermiculite, then were watered with 1000-fold diluted Hyponex (Osaka, Japan). They were photographed after the numbers of days indicated in Figures 2 and 4.

RNA gel blot analysis. *Arabidopsis* was grown on GM agar plates for 3 weeks and exposed to dehydration, high-salt, cold-stress, and ABA treatments, as described previously[19]. The plants were subjected to stress treatments for 5 h and then frozen in liquid nitrogen for further analyses. Isolation of total RNA and northern blot hybridization were performed as described previously[19]. The DNA fragments of the full-length *DREB1A*, *RD17*, *ERD10*, *AtP5CS*, *ERD1*, and *RD22* cDNAs and the 3′-terminal specific DNA fragments of *rd29A* and *rd29B* were used as probes. Probes for the *kin1*, *cor6.6*, and *cor15a* genes were obtained by PCR from cDNAs prepared from ABA-treated *Arabidopsis* plants.

Freezing, drought, and high-salt stress tolerance of the transgenic plants. Plants were grown in 9 cm pots filled with a 1:1 mixture of perlite and vermiculite. They were grown under continuous illumination of approximately 2500 lux at 22°C. Separate samples of the 3-week-old plants were exposed to freezing and drought stresses. Freezing stress was conducted by exposing the plants to -6°C temperatures for 2 days, then returning to 22°C for 5 days. Drought stress was conducted by withholding water for 2 weeks. High-salt stress was created by soaking plants that were grown on agar plates and gently pulled out of the growing medium in 600 mM NaCl solution for 2 h. The plants were then transferred to pots under normal growing conditions for 3 weeks. The numbers of plants that survived and continued to grow were counted. The statistical significance of the values was determined using chi-squared test.

Acknowledgments

The authors thank Satomi Yoshida, Atsuko Konishi, and Ekuko Ohgawara for their excellent technical assistance. This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences, which awarded a grant to K.Y.-S. This work was also supported in part by the Special Coordination Fund of the Science and Technology Agency, by the Hunan Frontier Science Program, and by a grant in aid from Japan's Ministry of Education, Science, and Culture to K.S.

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